

(19) World Intellectual Property Organization  
International Bureau



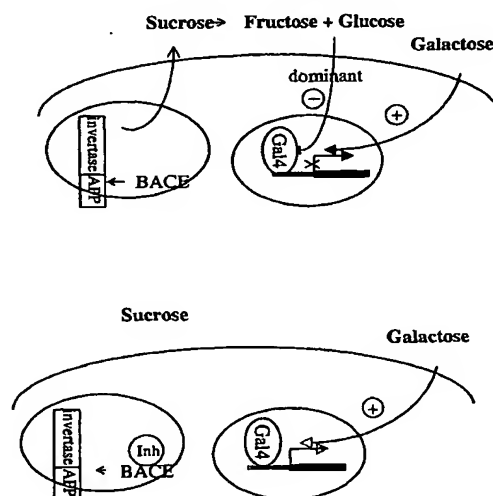
(43) International Publication Date  
23 October 2003 (23.10.2003)

PCT

(10) International Publication Number  
**WO 03/087842 A1**

- (51) International Patent Classification<sup>7</sup>: **G01N 33/68**, 33/50, C12Q 1/68, 1/02
- (21) International Application Number: **PCT/IB02/01342**
- (22) International Filing Date: **18 April 2002 (18.04.2002)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (71) Applicant (for all designated States except US): **ESBAT-ECH AG** [CH/CH]; Wagistrasse 21, CH-8952 Schlieren (CH).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **BARBERIS, Alcide** [CH/CH]; Allenmoosstrasse 64, CH-8057 Zürich (CH). **MIDDENDORP, Oliver, Michael** [CH/CH]; Hermann Götz Strasse 4, CH-8400 Winterthur (CH). **LÜTHI, Urs** [CH/CH]; Handgasse 2, CH-8180 Bülach (CH).
- (74) Agent: **E. BLUM & CO.**; Vorderberg 11, CH-8004 Zurich (CH).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR THE IDENTIFICATION OF MODULATORS OF A SECRETASE ACTIVITY



(57) Abstract: The present invention relates to a cellular screening method for the identification of modulators of secretases. Said method comprises contacting suitable host cells with a test substance wherein said suitable host cells comprise: a) a fusion protein comprising a secretory protein, a membrane anchor domain and a secretase cleavage sequence, b) a protein comprising a secretase activity recognising said cleavage sequence of said fusion protein and c) at least one reporter gene under control of a transcriptional activation system wherein said transcriptional activation system is regulated by the release of said secretory protein from said fusion protein by said secretase activity and its subsequent secretion then culturing said cells under suitable conditions such that said reporter gene allowing detection and/or survival of cells is only expressed or repressed in a manner that is dependent on an altered secretase activity due to said test substance.

**Method for the identification of modulators  
of a secretase activity**

Technical Field

5           The present invention relates to a *in vivo*  
method for the identification of modulators of secretase  
activities.

Background Art

10           Protein secretion is central to the proper  
development and function of eukaryotic organisms. Moreo-  
ver, several pathophysiological processes such as neuro-  
degeneration, oncogenesis, apoptosis and inflammation are  
associated with the malfunction or aberrant regulation of  
15 protein secretion. It has become clear that there is no  
single biosynthetic mechanism common to all secretory  
proteins. Secretion of proteins can occur through either  
the regulated or constitutive pathways and, in some cell  
types, this secretion can be polarized to distinct cellu-  
20 lar domains. An increasing number of proteins are now  
recognized as being derived from integral membrane pro-  
teins of type I and type II topology and, in this case,  
the secretory event involves their selective post-  
translational hydrolysis from the cell surface. This se-  
25 cretion is catalyzed by proteases known as secretases.  
The cleavage of membrane proteins generally occurs near  
the extracellular face of the membrane, although in some  
cases it has been shown also to occur within the trans-  
membrane domain. Proteins secreted in this fashion in-  
30 clude membrane receptors and receptor ligands, ectoen-  
zymes, cell adhesion molecules and others. Examples of  
protein secretion through the action of secretases in-  
clude the vasoregulatory enzyme ACE (angiotensin convert-  
ing enzyme), the tumor necrosis factor (TNF) ligand and  
35 receptor superfamilies, the transforming growth factor- $\alpha$ ,  
certain cytokine receptors, the Alzheimer's amyloid pre-  
cursor protein (APP) and others (7).

The involvement of secretases in the development of human diseases makes them potential drug target candidates in a variety of disease areas, including anti-cancer drugs, cardiovascular drugs, anti-neurodegenerative drugs and anti-inflammatory drugs.

In the past, different *in vitro/in vivo* and biochemical methods have been used to identify modulators of secretase enzymes.

US patent No. 5,942,400 and WO 96/40885 disclose *in vitro* methods for screening for candidate drugs for the ability to inhibit the activity of beta-secretase. Said methods are based on the detection of APP cleavage products using specific antibodies.

WO 98/13488 describes a method for determining the activity of modulators of APP secretases that are active in cultured human cells. Said method involves transfection of tissue-culture cells with vectors that express cleavable reporter proteins. Upon cleavage by the endogenous secretases, a reporter domain is secreted and can be detected using standard biochemical and immunological methods.

WO 01/49871 discloses an *in vivo* process for finding substances which specifically inhibit  $\gamma$ -secretase. Said process encompasses cells expressing a secretase activity and a fusion protein which comprises the substrate of said secretase with the specific cleavage site and a reporter. Said cells are contacted with a test substance and the quantity of reporter cleaved is either measured directly or indirectly. Suitable reporter proteins allowing direct detection are GFP, luciferase,  $\beta$ -galactosidase and secreted alkaline phosphatase. In case of an indirect detection of the reporter, the cleaved reporter is a transcription factor or part of a transcription factor which migrates into the nucleus and induces expression of a reporter e.g. luciferase.

Although the prior art already discloses screening methods for secretase modulators, there is a

need for improved reliable *in vivo* screening systems for the identification of modulators of a secretase activity.

5                    Disclosure of the Invention

Hence, it is a general object of the present invention to provide a method for the identification/isolation of modulators of a secretase activity. Said  
10 method is defined by the following steps:

suitable eukaryotic host cells that are contacted with a test substance wherein said suitable host cells comprise:

- 15            a) a fusion protein comprising a secretory protein, a membrane anchor domain and at least one secretase cleavage sequence,
- b) a protein comprising a secretase activity recognising said cleavage sequence of said fusion protein and
- 20            c) at least one reporter gene under control of a transcriptional activation system wherein said transcriptional activation system is regulated by the release of said secretory protein from said fusion protein and its subsequent secretion

25            then culturing said cells under suitable conditions such that said reporter gene allowing detection and/or survival of cells is only expressed or repressed in a manner that is dependent on an altered secretase activity due to said test substance.

30            In a preferred embodiment, the present invention relates to a method for the identification of a secretase inhibitor. Said method is characterised in that a reduced or no release of said secretory protein from said fusion protein due to a reduced/inhibited secretase activity leads to a reduced or no secretion of said secretory protein and to the induction of expression of said  
35 reporter gene. The induction of expression of said re-

porter gene allows under suitable culturing conditions the detection and/or survival of cells which are in contact with a test compound having an inhibitory effect on the secretase activity.

5           Said reporter gene is preferably selected from genes conferring antibiotic resistance, genes complementing auxotrophies and genes encoding reporter molecules with an activity that can be detected by colorimetric or fluorescent methods such as genes selected from  
10 the group consisting of: lacZ, Luciferase gene, green fluorescence protein gene and chloramphenicol acetyl transferase gene.

          In another preferred embodiment, the present invention relates to a method for the identification of  
15 stimulators of a secretase activity. Said method is characterised in that the release of said secretory protein from said fusion protein due to an enhanced secretase activity leads to repression of said reporter gene expression thereby allowing detection and/or survival of cells  
20 which are in contact with a test substance having an stimulating effect on the secretase activity. Appropriate culturing conditions of the cells must be used such that said fusion protein is not efficiently cleaved by the secretase in the absence of said test substance having a  
25 stimulating effect on the secretase activity.

          Said reporter gene is preferably selected from genes such as CYH2 or CAN1 conferring sensitivity to a chemical.

          In a further preferred embodiment of the invention said suitable host cells comprise a second reporter gene that is selected from the group consisting of:

35           a) genes encoding reporter molecules with an activity that can be detected by colorimetric or fluorescent methods such as genes selected from the group consisting of: lacZ, luciferase gene, green fluores-

cence gene (GFP) and chloramphenicol acetyl transferase gene (CAT),

b) genes conferring antibiotic resistance,

c) genes conferring sensitivity to a chemical and

5 d) genes complementing auxotrophies.

Illustrative examples of suitable reporter genes that can be used in the present invention are mentioned above.

In a method of the present invention any eukaryotic cell can be used, preferably a yeast cell.

10 The term membrane anchor domain as used herein refers to molecules and/or protein domains which are responsible for the membrane association of a protein and includes e.g. transmembrane domains and GPI anchors.

The term secretory protein as used herein encompasses polypeptides or fragments thereof which are destined for export. Said secretory protein has preferably an enzymatic activity, more preferably it is a protein with invertase activity or functional fragments of a protein with invertase activity. An especially preferred  
15 invertase is a yeast invertase or functional fragments thereof. Yet it is obvious for the man skilled in the art that other secretory proteins can be used in a method of the present invention.

Any recognition sequence of a known secretase  
25 can be used for the construction of said fusion protein of the present invention. Preferred secretase recognition sites are selected from the  $\beta$  site and the  $\alpha$  site of the human amyloid precursor protein (APP) and the S2 site of Notch 1 protein. A much preferred recognition site is the  
30  $\beta$  site of human APP in which the Lys595Asn and Met596Leu changes were introduced (Swedish APP mutant).

In a much preferred embodiment of the invention said fusion protein comprises amino acid residues 1-532 of yeast invertase, amino acid residues 590-695 of  
35 human APP and optionally an ER retention signal.

In a further preferred embodiments said fusion protein comprises amino acid residues 1-532 of yeast

invertase, amino acid residues 1714-1876 of human Notch 1 and optionally an ER retention signal.

Said fusion protein can be expressed from an extrachromosomal gene construct e.g. from an episomal  
5 vector enabling expression of the fusion protein in a host cell. Preferably the nucleic acid construct encoding the fusion protein is integrated into the genome of the host cell. The nucleic acid can be introduced into the cell by any transfection method leading to uptake of the  
10 nucleic acid sequence into the cell. Such methods are known to the man skilled in the art and are e.g. described in Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory, 2001).

The term secretase activity as used herein  
15 encompasses proteolytic enzymes or functional fragments thereof which cleave membrane associated proteins, integral membrane proteins of type I and type II topology.

In a preferred embodiment said protein comprising a secretase activity further comprises an ER sig-  
20 nal sequence.

Preferred secretase activities for the present invention are selected from  $\beta$ -secretase and  $\alpha$ -secretase of human APP.

In a preferred embodiment of the invention  
25 said protein comprising a  $\beta$ -secretase activity further comprises an ER signal sequence and amino acid residues 616-695 of human APP.

The protein comprising said secretase activity can be expressed endogenously by the host cell or it  
30 can be encoded by a nucleic acid construct e.g. an episomal expression vector or by a nucleic acid construct that is stably integrated into the genome of the host cell.

Another object of the present invention are compounds identified by a method of the present inven-  
35 tion.

#### Brief Description of the Drawings

The invention will be better understood and objects other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such description makes reference to the annexed drawings, wherein:

Fig. 1 shows the primary structure and cellular processing of APP. APP is processed by a sequential cleavage reaction during trafficking through the secretory pathway. The  $\alpha$ -secretase pathway is the preferred, constitutive pathway releasing, in combination with the  $\gamma$ -secretase, a non-toxic p3 fragment. In Alzheimer patients, the  $\alpha$ -secretase pathway is competed by the amyloidogenic  $\beta$ -secretase pathway, where, upon  $\gamma$ -secretase cleavage, secretion of the A $\beta$ -peptide leads to formation of extracellular plaques;

Fig. 2A shows the invertase selection system. A portion of APP harboring the  $\alpha$ - and the  $\beta$ -site is fused to the enzyme invertase. The  $\beta$ -secretase (BACE) is co-expressed with the invertase-APP fusion protein. Cleavage of the invertase-APP reporter at the  $\beta$ -site liberates the invertase. Secretion of the invertase leads to cleavage of sucrose into glucose and fructose, thus enabling growth on sucrose plates;

Fig. 2B shows a schematic drawing of the invertase-APP reporter. The invertase-APP reporter was constructed by fusing the full-length invertase (aa 1-532) to a portion of APP (aa 590-695) harboring the secretase sites, the transmembrane domain and the cytoplasmic tail. Fusion of the ER retention signal DEKKMP (Seq. Id. No. 1) to the C-term further retards growth of clones that do not express an active secretase;

Fig. 2C shows a schematic drawing of the engineered BACE. The signal sequence of BACE was replaced by the SUC2 signal sequence (aa 1-19) to ensure efficient translocation into the yeast ER. To achieve colocalization of BACE with the invertase-APP fusion protein, the C-term of BACE was substituted by a portion of APP har-



boring the transmembrane domain and the cytosolic tail (aa 616-695);

Fig. 2D shows that BACE enhances growth on sucrose plates. The invertase-APP fusion protein harboring the Swedish mutation was co-expressed with the indicated constructs [BACE, empty vector or Yap3p (a yeast secretase that specifically cleaves APP at the  $\alpha$ -site)] in a yeast strain deficient for the suc2 gene and the endogenous  $\alpha$ -secretases mkc7 and yap3. Growth on sucrose plates was monitored after 2.5 days and 3 days, respectively;

Fig. 3 shows principles of a cellular selection system to identify modulators of secretases. Fig. 3A-C show schematic drawings of yeast cells. The reporter gene is either the LacZ or different selectable marker genes, that can act either positively or negatively on growth. These genes are under the control of the Gal1/10 promoter (denoted by the GAL4 transcription factor);

Fig. 3A shows default readout (without BACE): The reporter gene is induced. Biscr1 cells harboring the invertase-APP fusion protein in the absence of any secretase activity express the reporter gene when grown on 5% sucrose/2% galactose. Galactose activates transcription of the reporter gene, whereas sucrose is inert to the system in the absence of secreted invertase;

Fig. 3B shows that in the presence of an active BACE the reporter gene is repressed. Cleavage of the invertase-APP(Sw) by the  $\beta$ -secretase leads to liberation and subsequent secretion of invertase. In the periplasm, invertase hydrolyses sucrose into glucose and fructose. The yielded glucose is taken up by the cell where it dominantly represses transcription of the reporter system;

Fig. 3C shows that in the presence of an inhibited BACE the reporter gene is induced. If the action of BACE is blocked by a potent inhibitor the readout of

the system is the same as in the absence of BACE (described in 3A);

Fig. 4A shows a schematic representation of the reporter gene. The HIS3 open reading frame (ORF) was fused to the 47 N-terminal amino acids of GAL10. The expression of the GAL10-HIS3 fusion protein is under the control of the GAL10 promoter, the expression of the LacZ ORF is controlled by the GAL1 promoter. The arrows indicate the transcription start points. The boxes labeled I, II, III and IV represent GAL4 binding sites;

Fig. 4B shows LacZ assays. Biscresol cells harboring the indicated constructs were cultivated in 5% sucrose 2% galactose -ura -trp drop out medium. Expression of the LacZ reporter gene was quantified using the  $\beta$ -galactosidase substrate ONPG. The columns represent the average  $\beta$ -galactosidase units of three samples each. Error bars are indicated. Invertase: soluble and secreted invertase; Invertase-APP: displayed in figure 2B; Invertase-APP(Sw): the same as invertase-APP, but harboring the Swedish mutation at the  $\beta$ -site; BACE: displayed in figure 2C; BACEi: the same as BACE, but harboring a point mutation in the active center, which renders the enzyme inactive. The constructs named "empty" only carry the yeast marker genes necessary to grow in the drop out medium,

Fig. 4C shows liquid growth assays. Biscresol cells harboring the indicated constructs were cultivated in non-selective -ura -trp 2% glucose medium as well as in selective -his -ura -trp 5% sucrose 2% galactose medium. After 24 h, the cell density of the cultures was determined by measuring the optical density (OD600). The cell density in the selective medium is an indicator for the expression of the HIS3 gene. For the explanation of the different constructs, see legend of figure 4C. For the selective cultures, the average OD600 of three cultures is displayed. The standard deviation is indicated by an error bar.

Fig. 5 shows that cleavage of invertase-APP by the yeast secretase Yap3p can be monitored by the cellular system to identify modulators of secretases. Induction of reporter gene expression (LacZ) was measured in a  
5 LacZ assay.

Fig. 6 shows a schematic drawing of the invertase-Notch 1 reporter. The invertase-Notch 1 reporter was constructed by fusing the full-length invertase (aa 1-532) to a portion of Notch 1 (aa 1714-1876) harboring  
10 the S2 site of Notch 1 protein, the transmembrane domain and the cytoplasmic tail. Fusion of the ER retention signal DEKKMP (Seq. Id. No. 1) to the C-term further retards growth of clones that do not express an active secretase and

Fig. 7 shows that cleavage of invertase-Notch by the yeast  $\alpha$ -secretase Yap3p can be monitored by the cellular system to identify modulators of secretases. Induction of reporter gene expression (LacZ) was measured  
15 in a LacZ assay.

20

### Modes for Carrying Out the Invention

A reporter gene for the use in the present  
25 invention is under control of a transcriptional activation system. A preferred transcriptional activation system is a GAL gene regulatory system, more preferably a yeast GAL gene regulatory system.

In a preferred embodiment of the invention  
30 said reporter gene is under control of the yeast GAL1-10 gene regulatory region.

The use of said system in a preferred embodiment of the present invention has the following theoretical background:

35 The preferred carbohydrate of yeast cells is glucose since it can be metabolized directly. Other carbohydrates are converted to the glycolytic substrate glu-

cose-1-phosphate in several steps. Addition of galactose to yeast cultures grown on glycerol induces expression of the GAL genes at least 1000-fold. If glucose is added to the galactose-containing media, the GAL genes are induced  
5 to only 1% of the levels obtained with galactose alone. This phenomenon is known as glucose repression. GAL2, GAL1-GAL7-GAL10, and MEL1 are the GAL structural genes. Their products transport galactose into the cell and convert it to glucose-1-phosphate. The major regulatory proteins of this system are the products of the genes GAL3,  
10 GAL4, and GAL80. Gal4p, the product of the GAL4 gene, is a transcriptional activator that binds specific regulatory DNA elements called UAS<sub>G</sub>, which are present near the promoter regions of the galactose inducible genes. Its  
15 activity is required to stimulate expression of the GAL genes in the presence of galactose. Gal80p is a direct repressor of Gal4p, while Gal3p mediates the galactose-dependent release of Gal80p inhibition of Gal4p (for review see [5]).

20         The glucose repression is exerted through several mechanisms [5]. Here, we focus on the regulation of the divergently oriented GAL1-10 genes, whose regulatory region was used for the experiments described below. The GAL1-10 regulatory region contains four UAS<sub>G</sub> elements  
25 located between the divergent GAL1 and GAL10 promoters. These UAS<sub>G</sub> elements are bound by Gal4p in a cooperative manner. Due to this cooperativity, the system is rather sensitive towards changes in the concentration of Gal4p, which is reduced 3- to 5-fold in the presence of glucose.  
30 Gal80p significantly contributes to glucose repression of the GAL1-10 genes by binding to Gal4p to mask its activation domain, thereby preventing expression of these GAL genes [6]. The GAL1 promoter contains an additional regulatory element, which is the binding site for the repressor Mig1p. The activity of this repressor is also  
35 regulated by glucose. (A Mig1p site is also present in the GAL4 promoter.)

The construction of suitable host cells and the other molecular biological reagents for the use in the present invention e.g. fusion protein constructs can be done using standard molecular biology techniques as  
5 described e.g. in Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory, 2001).

The man skilled in the art is as well able to determine suitable culturing conditions allowing the de-  
10 tection and/or survival of the used cells. Said conditions are dependent on the used genetic constructs and the host cells.

There are at least three different categories of compounds that can be screened by a screening method  
15 of the present invention: chemical libraries, natural product libraries and combinatorial libraries. Chemical libraries consist of structural analogues of known compounds. Natural product libraries are collections of microorganism, animals, plants or marine organisms which  
20 are used to create mixtures for screening by for example fermentation and extraction of broths from soil, plant or marine microorganisms or extraction of plants or marine organisms. Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic com-  
25 pounds as a mixture. They are relatively easy to prepare e.g. by traditional synthesis methods, PCR or cloning.

In a screening test of the present invention a test compound can e.g. be added to the culture medium of the host cells or it can be expressed by the host  
30 cells e.g. from an expression construct. The expression of test substances within the host cells is e.g. suitable for the screening of peptide libraries.

The present invention is now further illustrated by means of examples.

Reconstitution of secretase activities in  
yeast

To monitor BACE activity within yeast cells,  
5 we made use of the invertase reporter system described in  
our patent application WO 01/75088, Title: "Method for  
identification of polypeptides with protease activity" .

Secretion of invertase enables yeast to use  
sucrose as a carbon source. The invertase hydrolyzes su-  
10 crose to yield fructose and glucose. For the reporter  
system described in our patent application WO 01/75088,  
the endogenous gene encoding the invertase (the SUC2  
gene) was knocked out. A recombinant invertase was fused  
to the N-terminus of a portion of APP harboring the  
15 transmembrane domain as well as the  $\alpha$ - and  $\beta$ -sites (resi-  
dues 590-695). In addition, an ER retention signal was  
added to the C-terminus of this fusion construct (Fig.  
2A, B). These modifications prevent invertase from being  
secreted and, consequently, the yeast cells are unable to  
20 efficiently grow on plates containing sucrose as the only  
carbohydrate. The system described here is based on the  
observation that, upon cleavage of the APP portion at the  
 $\alpha$ - or  $\beta$ -site, the invertase is liberated and secreted in  
the extracellular environment, where it hydrolyzes su-  
25 crose into fructose and glucose.

There are two yeast endogenous secretases de-  
scribed in the literature which can cleave APP at the  $\alpha$ -  
site: Yap3p and Mkc7 [1, 2]. Since these proteins have  $\alpha$ -  
secretase activity that constitutively cleaves APP, their  
30 respective genes had to be knocked out in order to inves-  
tigate  $\beta$ -secretase activity in yeast using the invertase  
reporter system described in WO 01/75088.

To detect APP-specific BACE activity within  
yeast cells, the protein had to be modified. Since trans-  
35 membrane and cytosolic sequences contribute to the sub-  
cellular localization of transmembrane proteins, the  
transmembrane and cytosolic portion of BACE was substi-

tuted by the transmembrane and cytosolic portion of APP. In this way, co-localization of the enzyme with its substrate was facilitated. To ensure efficient translocation into the yeast ER, the signal sequence of BACE was substituted with the SUC2-signal peptide at the N-terminus of the protein (Fig. 2C). Since BACE cleaves the APP wild type less efficiently than the so-called Swedish APP mutant [3], the  $\beta$ -site of the invertase-APP (590-695) reporter construct was altered such as to introduce the Lys595Asn and Met596Leu changes indicated as invertase-APP (Sw). Figure 2D shows the growth effect on sucrose plates due to cleavage of the invertase-APP (Sw) reporter protein by BACE or Yap3p.

15

#### Cellular system to screen for secretase inhibitors

In order to establish the described cellular system to screen for BACE inhibitors, we combined the BACE-dependent invertase secretion system with the endogenous GAL gene regulatory network.

A reporter construct was cloned which expresses the LacZ gene under the control of the GAL1 promoter and the divergently oriented HIS3 gene under the control of the GAL10 promoter. As in the case of the endogenous GAL1 and GAL10 genes, four UAS<sub>G</sub> elements are located between these two promoters. This reporter construct was integrated in yeast cells deficient for suc2, mkc7 and yap3 to create the strain Biscr1. If this strain is grown in the presence of galactose, expression of the LacZ gene and the HIS3 gene is induced by the UAS<sub>G</sub>-binding Gal4p activator. As in the case of the endogenous GAL1 and GAL10 genes, these reporter genes become dominantly repressed upon addition of glucose to the medium. The product of the HIS3 gene is needed for growth on histidine-depleted (-his) medium. Consequently, Biscr1 can only grow on -his media that contain galactose

but no glucose. The HIS3 reporter gene of the strain Biscrel, which lacks the endogenous invertase activity, is also induced by Gal4p when these cells grow in the presence of sucrose together with galactose (Fig 3A). However, if Biscrel is allowed to secrete the invertase, glucose is generated by the hydrolysis of sucrose. As consequences, the newly generated glucose dominantly represses the HIS3 gene expressed from the GAL10 promoter and growth on -his medium is inhibited.

Biscrel cultured on sucrose and galactose and expressing the invertase-APP (Sw) fusion protein provides a tool to select for inhibited BACE. Indeed, if BACE expressed in Biscrel is active, the membrane-anchored invertase-APP (Sw) fusion protein is cleaved at the  $\beta$  site and the invertase moiety is secreted. The secreted invertase hydrolyses sucrose into fructose and glucose, the latter of which represses the HIS3 and the LacZ reporter genes (Fig 3B). If BACE is inhibited, either by mutations or by an inhibiting compound, the invertase moiety remains anchored to the ER-membrane via the APP domain, the sucrose in the medium is not hydrolyzed, and expression of the HIS3 and LacZ genes is induced by galactose (Fig 3C).

### Results

Expression of the product of the LacZ gene, a  $\beta$ -galactosidase, can be measured by using the substrate analogue o-nitro-phenyl- $\beta$ -galactopyranosid (ONPG). A cleavage product of ONPG has a yellow colour. OD measurement at 420 nm allows quantification of the reaction catalyzed by  $\beta$ -galactosidase, and indirectly of the LacZ-expression. This assay allowed us to investigate the effect of invertase-APP cleavage by BACE in the context of Biscrel.

Biscrel was transformed with plasmids expressing different  $\beta$ -secretase activities together with the invertase-APP (Sw) construct, or its wildtype variant



(invertase-APP). Empty plasmids and an invertase-expressing plasmid were used as negative and positive controls, respectively. Liquid cultures were grown in 5 % sucrose, 2 % galactose, 0.1 % glucose drop-out medium  
5 (The medium contained histidine for this assay as the cultures should grow equally). The maximal level of LacZ expression in this assay was obtained with transformants harboring two empty plasmids. In these cells there was no  
10 invertase activity, and consequently no glucose was produced in the presence of sucrose. In this way, values of  $>0.6$   $\beta$ -galactosidase units were measured (Fig. 4B, column 1). Expression of soluble and secreted invertase resulted in a strong repression of the reporter gene (Fig. 4B, column 2). Expression of the invertase-APP (Sw) fusion  
15 protein alone resulted in a  $\beta$ -galactosidase activity of 0.25 units (Fig. 4B, column 3). Expression of the invertase-APP wild-type protein resulted in a comparable level of LacZ gene activation (Fig. 4B, column 4). The 2-3-fold reduction in LacZ expression observed in the presence of  
20 these invertase fusion proteins is most likely due to the residual appearance of the invertase moiety at the surface of the cell. This could be due to a small portion of the fusion protein that reaches the cell surface, or to some residual endogenous secretase activity present in  
25 the cell despite the fact that the two major endogenous  $\alpha$ -secretases Yap3p and Mkc7p were knocked out.

Co-expression of BACE with the invertase-APP (Sw) fusion protein led to a significant reduction of  $\beta$ -galactosidase activity (0.026  $\beta$ -galactosidase units, Fig.  
30 4B, column 5), whereas co-expression with the construct harboring the APP wild-type sequence (invertase-APP) resulted in an only slight reduction (Fig. 4B, column 6). A catalytically inactive version of BACE (BACEi) behaved  
35 similarly as an empty plasmid when expressed together with either one of these fusion constructs (Fig. 4B, columns 7, 8).

These results show that the expression level of the reporter gene system is dependent on the presence of free invertase. Free invertase was generated either by direct expression of the natural SUC2 gene or by liberation from a membrane-bound fusion protein. The liberation and subsequent secretion of the invertase moiety only took place efficiently when the active BACE secretase was co-expressed with the invertase fusion protein harboring the Swedish mutation, thus underlying the specificity of the system.

The expression of the HIS3 gene was quantified by a growth assay in liquid medium. For this assay, Biscr1 cells were cultivated in liquid -his medium containing 5% sucrose and 2% galactose (selective conditions). Two-ml cultures were inoculated with equal amounts of cells transformed with the constructs of interest. Cell density was measured after 24 h. The same amounts of cells were used to inoculate cultures in non-selective medium. This control experiment showed that none of the constructs per se had an effect on cell growth under non-selective conditions (Fig. 4C, columns 1-4). The cell densities measured after growth in selective medium are displayed in figure 4C, columns 5-8. Biscr1 harboring two empty plasmids fully induced the HIS3 gene and could grow under selective conditions (Fig. 4C, column 5). The cell density of this culture was comparable to those of all the cultures grown in non-selective medium. The cells transformed with the invertase-APP (Sw) construct together with an empty plasmid or with the inactivated BACE grew to a density of about 75% of the one reached with two empty plasmids (Fig. 4C, column 6, 7). Expression of active BACE together with the invertase-APP (Sw) fusion protein reduced cell growth to about 10% of the values obtained with inactivated BACE (Fig. 4C, column 8). Therefore, this assay provides a tool to screen compound libraries to identify BACE inhibitors that are

active in a eukaryotic cellular environment and that do not negatively affect basic cellular functions.

The screening system is as well suitable to screen for modulators of other secretases. Figure 5 shows the results of such an experiment. Yap3p is a yeast secretase which cleaves APP at the  $\alpha$ -site. The experimental procedures were the same as described for fig. 4B. The mutated Yap3p as well as the wildtype version were provided on a yeast expression vector. Yap3p cleaves the invertase-APP fusion protein at the  $\alpha$ -site thereby liberating the invertase. The observed effect on LacZ expression is comparable to the effect observed when soluble and secreted invertase was expressed (last column).

To further investigate the general applicability of our system, a different target, namely the human Notch 1 protein, was fused to invertase (Fig. 6). The features of this new construct are identical to those of the invertase-APP fusion protein, except that the APP portion is replaced by the amino acids 1714-1876 of the Notch 1 protein (NCBI Protein Databank accession number AAG33848), which include the transmembrane domain. The invertase-Notch construct was co-expressed with Yap3p ( $\alpha$ -secretase) or with an empty vector in the Biscrel strain and a  $\beta$ -gal assay was performed. The results of this experiment are displayed in figure 7. The first column indicates the value obtained with the empty vector (0.4532  $\beta$ -gal units); the second column indicates the value obtained for Yap3p (0.0724  $\beta$ -gal units). The cleavage of the invertase-Notch fusion protein was confirmed by detecting the cleavage products in a Western blot (data not shown).

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

### References

1. Zhang, W., et al., *Characterization of*  
5 *beta-amyloid peptide precursor processing by the yeast*  
*Yap3 and Mkc7 proteases. Biochimica et Biophysica Acta,*  
1997. **1359**(2): p. 110-22.
2. Komano, H., et al., *Involvement of cell*  
*surface glycosyl-phosphatidylinositol-linked aspartyl*  
10 *proteases in alpha-secretase-type cleavage and ectodomain*  
*solubilization of human Alzheimer beta-amyloid precursor*  
*protein in yeast. J Biol Chem, 1998. 273(48): p. 31648-*  
51.
3. Citron, M., et al., *Mutation of the beta-*  
15 *amyloid precursor protein in familial Alzheimer's disease*  
*increases beta-protein production. Nature, 1992.*  
**360**(6405): p. 672-4.
4. Lohr, D., P. Venkov, and J. Zlatanova,  
*Transcriptional regulation in the yeast GAL gene family:*  
20 *a complex genetic network. The FASEB Journal, 1995. 9: p.*  
777-786.
5. Lamphier, M.S. and M. Ptashne, *Multiple*  
*mechanisms mediate glucose repression of the yeast GAL1*  
*gene. Proc. Natl. Acad. Sci. USA, 1992. 89: p. 5922-5926.*
- 25 6. Melcher, K. and H.E. Xu, *Gal80-Gal80 in-*  
*teraction on adjacent Gal4p binding sites is required for*  
*complete GAL gene repression. Embo J, 2001. 20(4): p.*  
841-851.
7. Hooper, N.M., Karran, E.H., Turner, A.J.  
30 *Membrane protein secretases Biochem. J, 1997. 321: 265-*  
279.

Claims

1. A method for the identification/isolation of modulators of a secretase activity wherein

5           suitable eukaryotic host cells are contacted with a test substance wherein said suitable host cells comprise:

10           a) a fusion protein comprising a secretory protein, a membrane anchor domain and a secretase cleavage sequence,

          b) a protein comprising a secretase activity recognising said cleavage sequence of said fusion protein and

15           c) at least one reporter gene under control of a transcriptional activation system wherein said transcriptional activation system is regulated by the release of said secretory protein from said fusion protein by said secretase activity and its subsequent secretion

20           then culturing said cells under suitable conditions such that said reporter gene allowing detection and/or survival of cells is only expressed or repressed in a manner that is dependent on an altered secretase activity due to said test substance.

25           2. Method for the isolation of a secretase inhibitor according to claim 1, wherein a reduced or no release of said secretory protein due to a reduced/ inhibited secretase activity leads to expression of said at least one reporter gene thereby allowing detection and/or

30 survival of cells under suitable culturing conditions.

          3. Method according to claim 1 or 2, wherein said at least one reporter gene is selected from genes conferring antibiotic resistance, genes encoding reporter molecules with an activity that can be detected by col-

35 orimetric or fluorescent methods and genes complementing auxotrophies, preferably a His3 gene.

4. Method for the identification of agonists of a secretase activity according to claim 1, wherein the release of said secretory protein due to an enhanced secretase activity leads to a reduced expression of said at least one reporter gene thereby allowing detection and/or survival of cells under suitable conditions.

5. Method according to claim 4, wherein said at least one reporter gene is selected from genes conferring sensitivity to a chemical.

6. Method according to anyone of the preceding claims, wherein said cells comprise a second reporter gene selected from the group consisting of:

- a) genes encoding reporter molecules with an activity that can be detected by colorimetric or fluorescent methods,
- b) genes conferring antibiotic resistance and genes conferring sensitivity to a chemical and
- c) genes complementing auxotrophies.

7. Method according to anyone of the preceding claims, wherein said cell is a yeast cell.

8. Method according to anyone of the preceding claims, wherein said secretory protein has an enzymatic activity, preferably a protein with invertase activity or functional fragments of a protein with invertase activity.

9. Method according to anyone of the preceding claims, wherein said secretory protein is a yeast invertase or functional fragments of a yeast invertase.

10. Method according to anyone of the preceding claims, wherein said secretase cleavage sequence is selected from the  $\beta$  site and the  $\alpha$  site of the human amyloid precursor protein and the S2 site of Notch 1 protein.

11. Method according to claim 10, wherein into said  $\beta$  site the Lys595Asn and Met596Leu changes were introduced.

12. Method according to anyone of the preceding claims, wherein said fusion protein comprises an ER retention signal.

13. Method according to anyone of the preceding claims, wherein said fusion protein comprises amino acid residues 1-532 of yeast invertase, amino acid residues 590-695 of human APP and an ER retention signal.

14. Method according to anyone of the preceding claims, wherein said fusion protein comprises amino acid residues 1-532 of yeast invertase, amino acid residues 1714-1876 of human Notch 1 and an ER retention signal.

15. Method according to anyone of the preceding claims, wherein a nucleic acid construct encoding said fusion protein is integrated into the genome of said host cell.

16. Method according to anyone of the preceding claims, wherein said protein comprising a secretase activity recognising said cleavage sequence of said fusion protein further comprises an ER signal sequence.

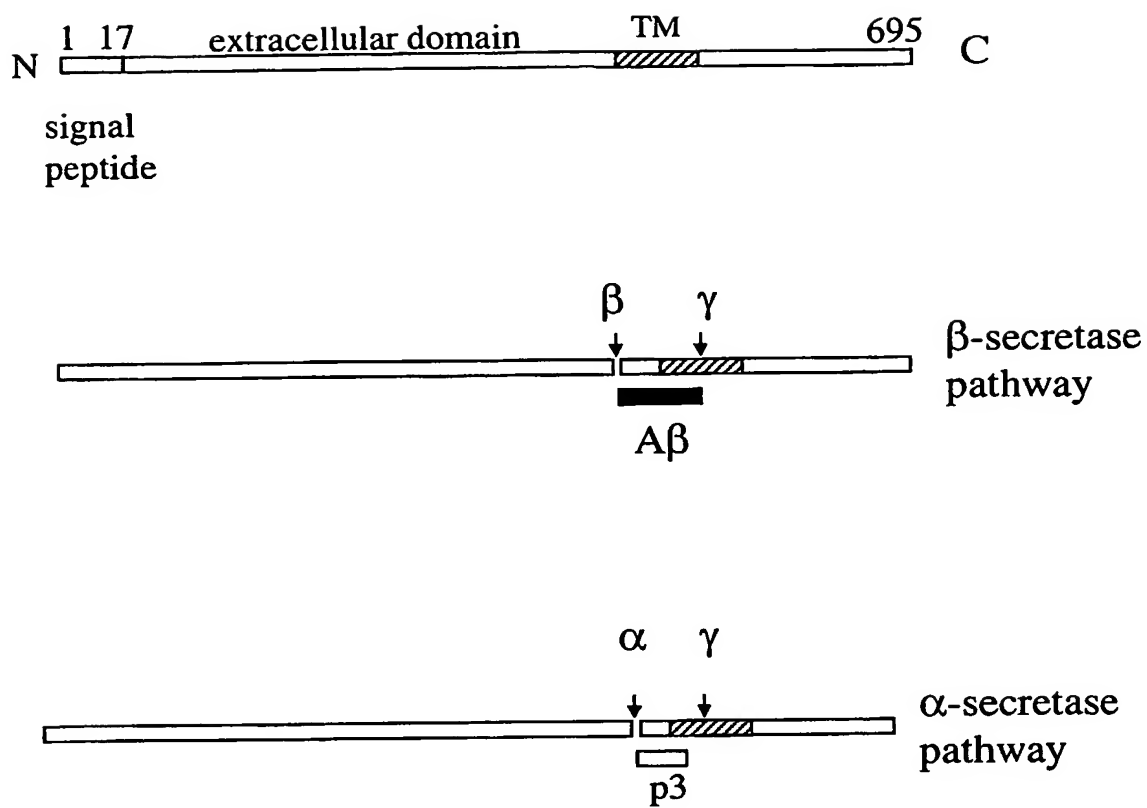
17. Method according to anyone of the preceding claims, wherein said secretase activity is selected from  $\beta$ -secretase and  $\alpha$ -secretase.

18. Method according to claim 16, wherein said protein comprising a  $\beta$ -secretase activity further comprises an ER signal sequence and amino acid residues 616-695 of human APP.

19. Method according to anyone of the preceding claims, wherein said transcriptional activation system comprises at least part of the yeast GAL gene regulatory system.

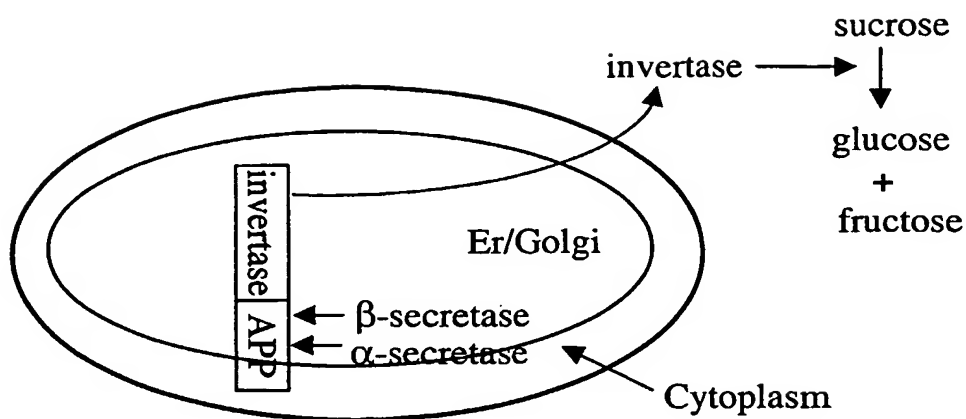
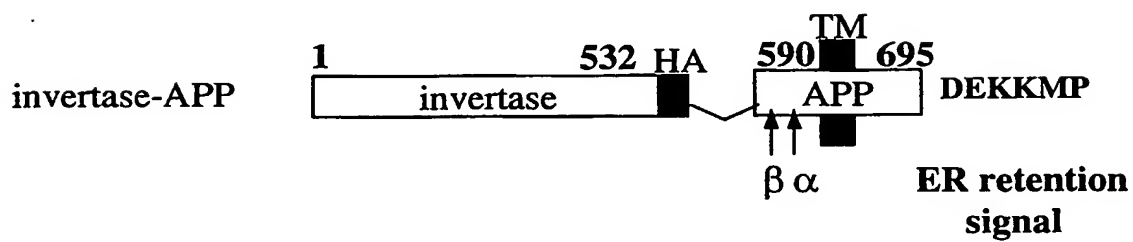
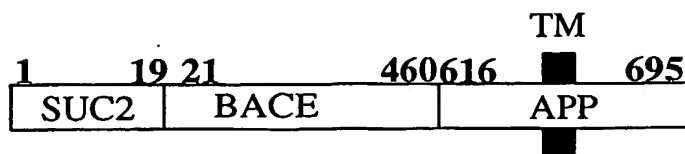
20. Method according to anyone of the preceding claims, wherein said reporter gene is under control of the yeast GAL1-10 regulatory region.

1/8

**Fig. 1**



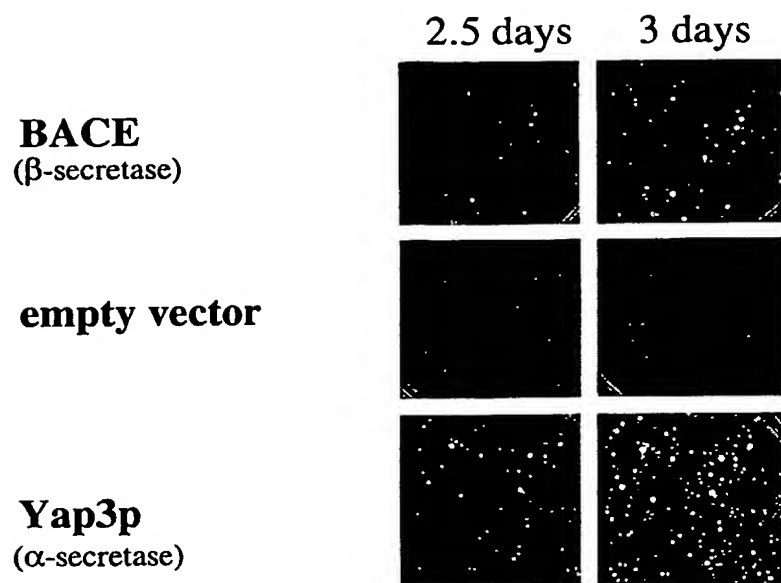
2/8

**Fig. 2 A****Fig. 2 B****Fig. 2 C**

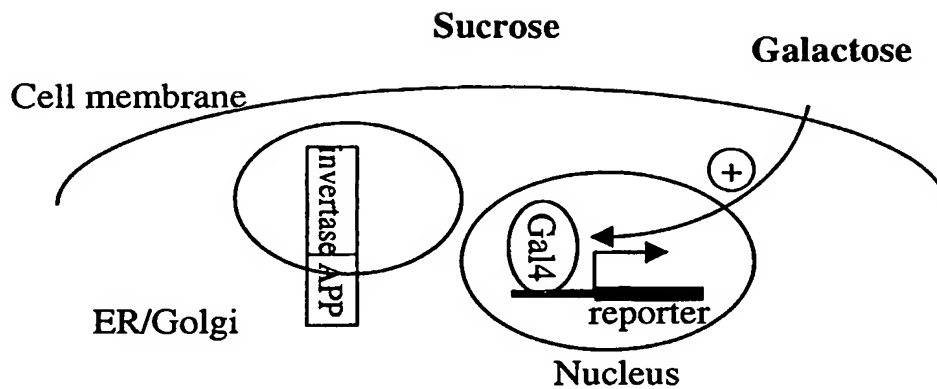
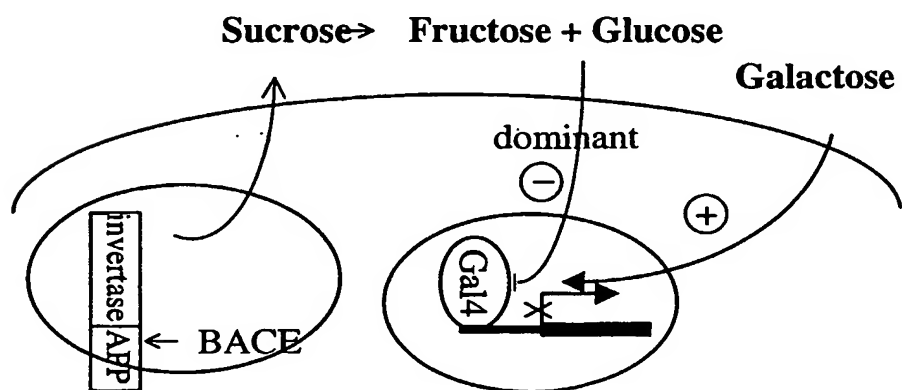
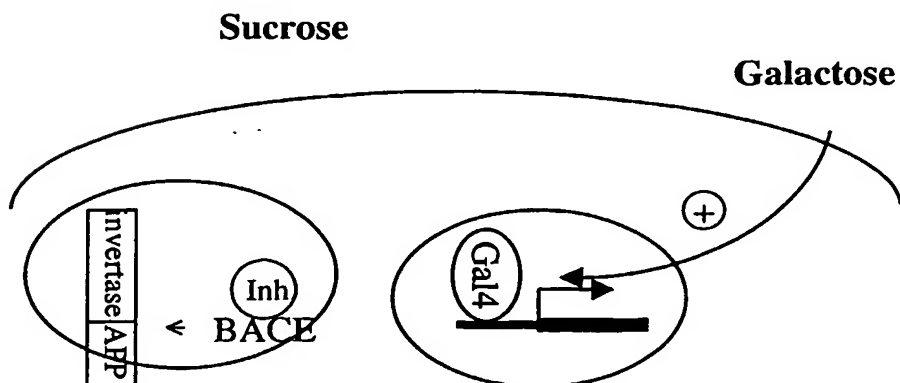
3/8

BEST AVAILABLE COPY

Fig. 2 D



4/8

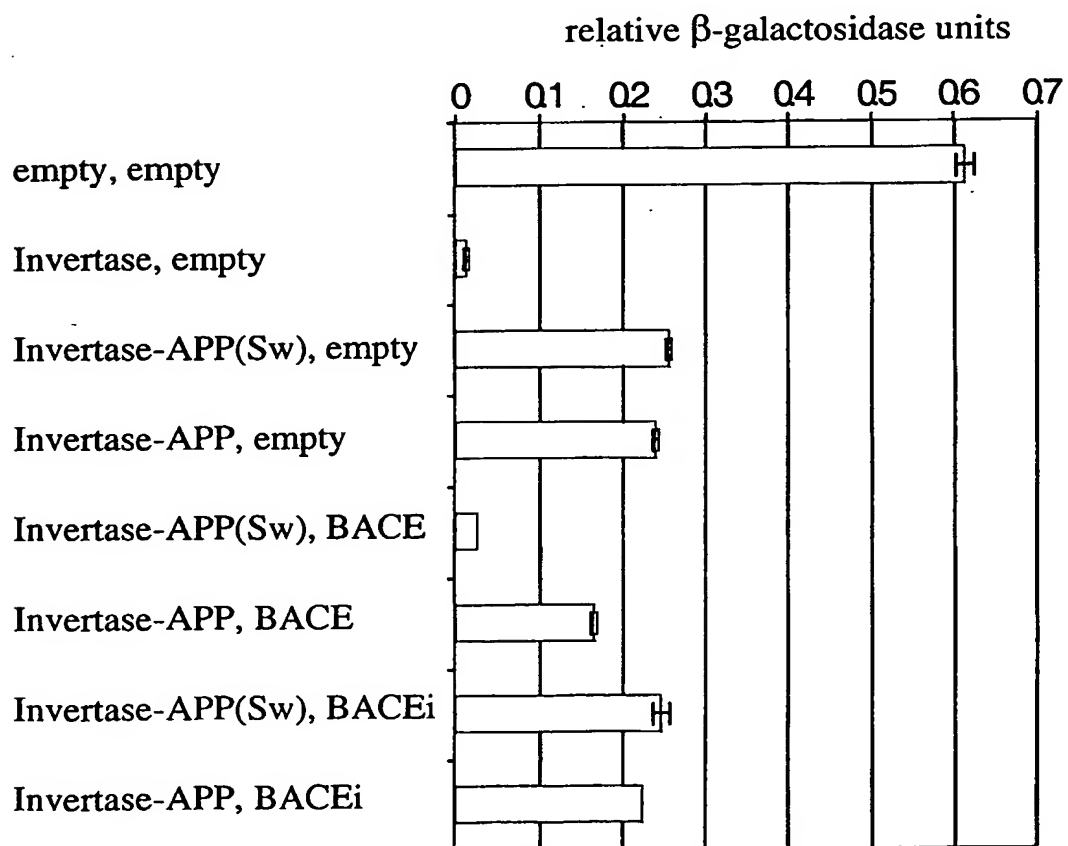
**Fig. 3 A****Fig. 3 B****Fig. 3 C**

5/8

Fig. 4 A

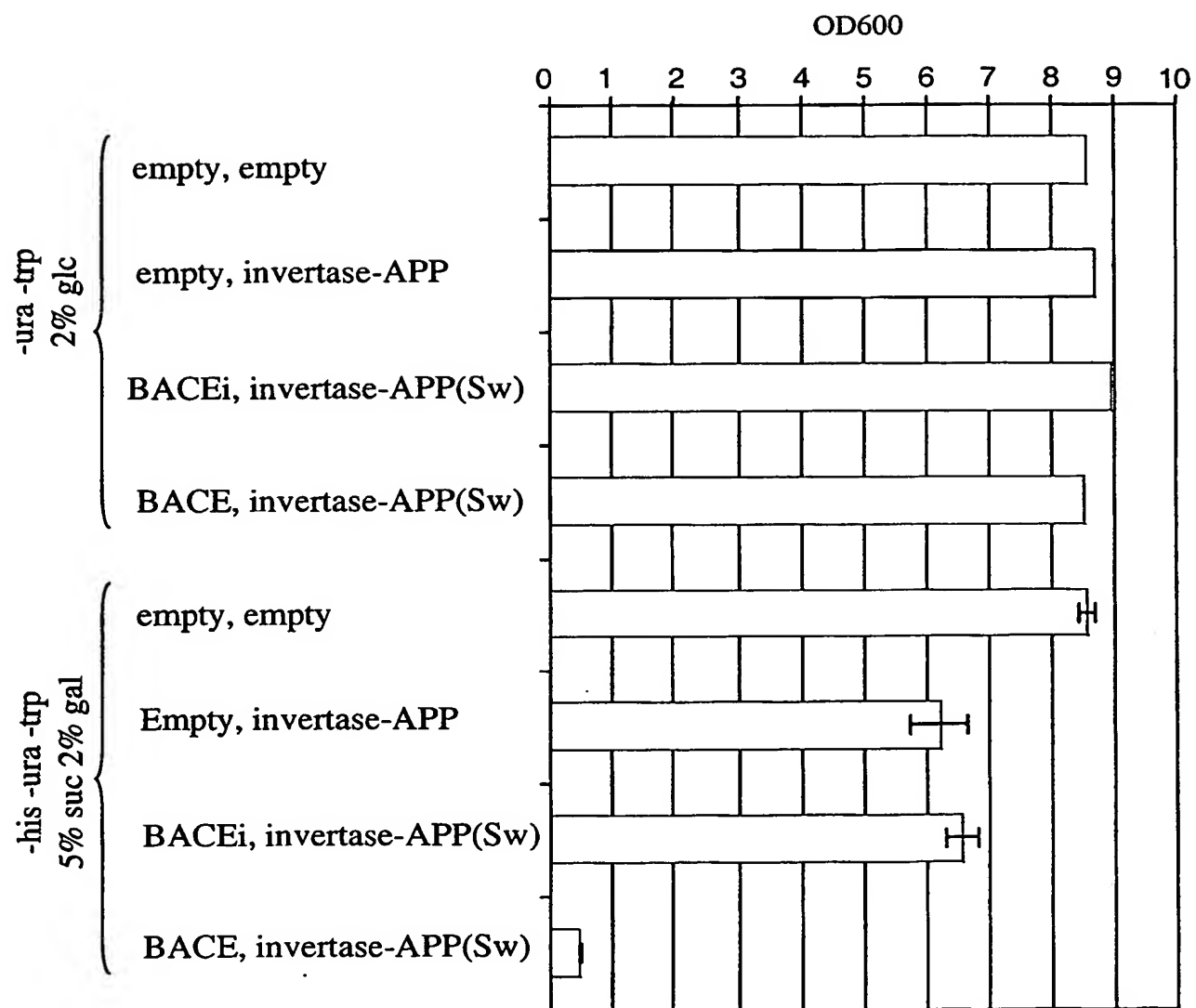


Fig. 4 B



6/8

Fig. 4 C



7/8

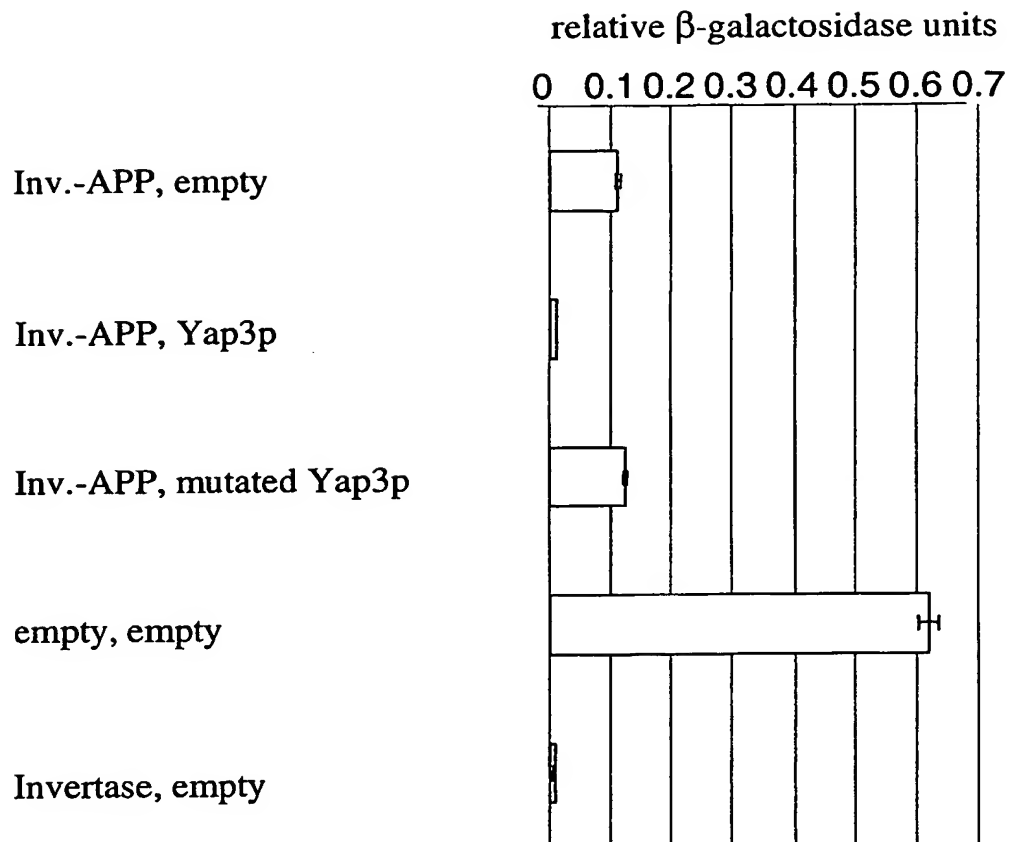
**Fig. 5**

Fig 6.

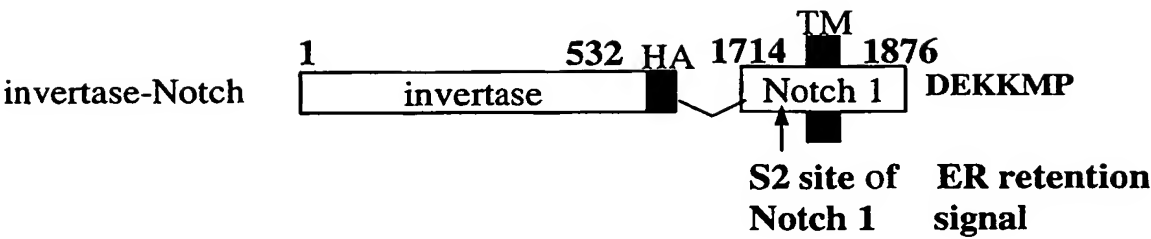
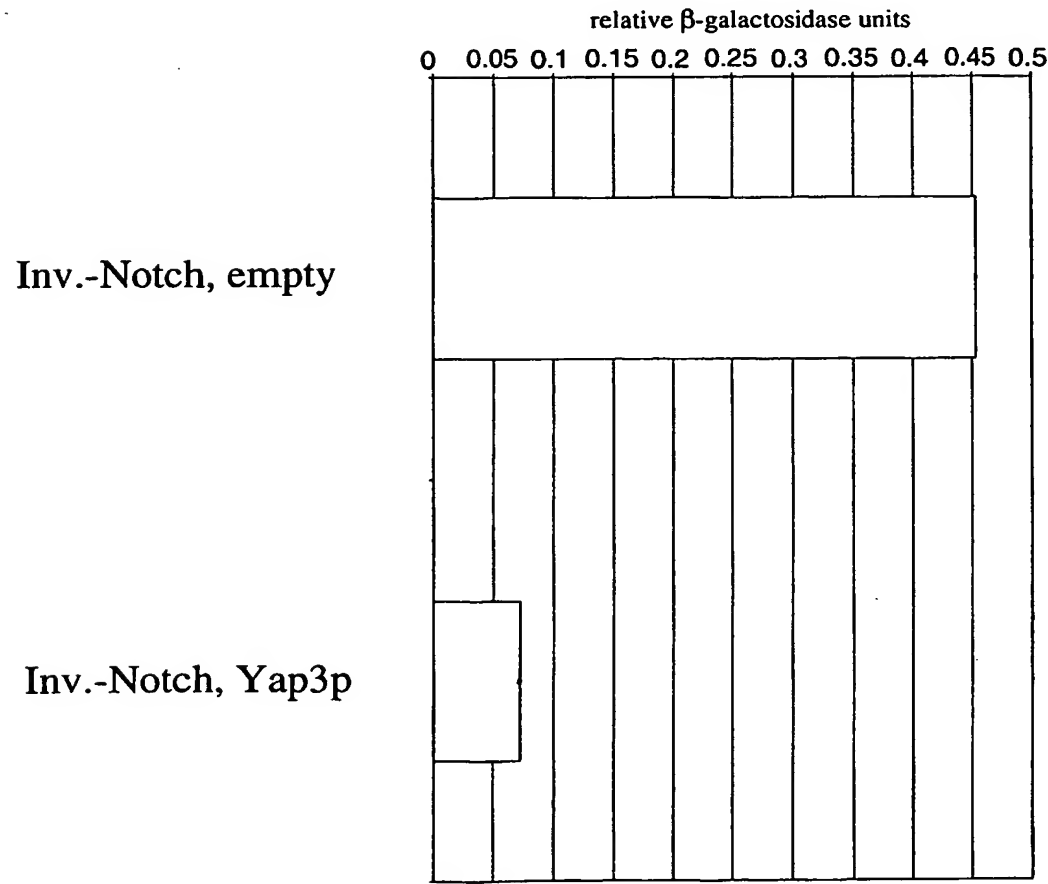


Fig. 7



## SEQUENCE LISTING

<110> ESBATech AG  
<120> Method for the identification of modulators of a secretase activity  
<130> 05606PC  
<160> 1  
<170> PatentIn version 3.1  
<210> 1  
<211> 6  
<212> PRT  
<213> Artificial sequence  
<220>  
<223> ER retention peptide  
<400> 1  
Asp Glu Lys Lys Met Pro  
1 5



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/12/01342

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/68 G01N33/50 C12Q1/68 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 75088 A (ESBATECH AG ; BARBERIS ALCIDE (CH); LUETHI URS (CH)) 11 October 2001 (2001-10-11) cited in the application the whole document ---	1-20
X	WO 00 34511 A (AVENTIS PHARMA GMBH) 15 June 2000 (2000-06-15) page 5, line 15, to page 8, line 8; page 11, line 23, to page 12, line 16; Figures 1 and 2 ---	1-4, 10, 12, 15, 19
X	US 2002/025508 A1 (FUCHS KLAUS ET AL) 28 February 2002 (2002-02-28) Abstract; pages 2-4; Figures 1 and 2; Example 3 --- -/--	1-4, 10, 12, 15, 19

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

13 February 2003

Date of mailing of the international search report

27/02/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Aguilera, M

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/02/01342

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 62897 A (SERNEELS LUTGARDE ;VLAAMS INTERUNIVERSITAIR INST (BE); ANNAERT WIM) 30 August 2001 (2001-08-30) Abstract; Figure 4A; Example 5 ---	1-4,10, 12,15,19
X	US 6 333 167 B1 (SHUEY DAVID J ET AL) 25 December 2001 (2001-12-25) Abstract; Figures 1 and 2; column 3, line 5, to column 4, line 19; column 8, line 47, to column 10, line 2 ---	1-4,10, 12,15,19
X	KARLSTROM HELENA ET AL: "A sensitive and quantitative assay for measuring cleavage of presenilin substrates." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 9, 1 March 2002 (2002-03-01), pages 6763-6766, XP002231028 March 1, 2002 ISSN: 0021-9258 the whole document ---	1-4,10, 12,15,19
Y	WO 02 06306 A (GURNEY MARK E ;BIENKOWSKI MICHAEL JEROME (US); HEINRIKSON ROBERT L) 24 January 2002 (2002-01-24) page 32, line 30 -page 34, line 31 page 8, line 26 -page 9, line 16 ---	1-20
Y	WO 98 13488 A (SCHERING AG ;DYRKS THOMAS (DE); HAERTEL MARION (DE); TURNER JONATH) 2 April 1998 (1998-04-02) cited in the application the whole document ---	1-20
Y	DIXON E P ET AL: "AN INVERSE MAMMALIAN TWO-HYBRID SYSTEM FOR BETA SECRETASE AND OTHERPROTEASES" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 249, no. 2, 1997, pages 239-241, XP000914815 ISSN: 0003-2697 the whole document ---	1-20

-/--

## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/92/01342

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEEHAN C ET AL: "SHEDDING OF THE LYMPHOCYTE L-SELECTIN ADHESION MOLECULE IS INHIBITED BY A HYDROXAMIC ACID-BASED PROTEASE INHIBITOR IDENTIFICATION WITH AN L-SELECTIN-ALKALINE PHOSPHATASE REPORTER" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 271, no. 12, 22 March 1996 (1996-03-22), pages 7019-7024, XP000616670 ISSN: 0021-9258 the whole document ---	1-20
Y	DE 199 20 514 A (BOEHRINGER INGELHEIM PHARMA) 16 November 2000 (2000-11-16) the whole document -----	1-20

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/02/01342

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 0175088	A	11-10-2001	AU	3833100 A	15-10-2001
			EP	1268755 A1	02-01-2003
			WO	0175088 A1	11-10-2001
WO 0034511	A	15-06-2000	DE	19856261 C1	30-03-2000
			AU	1968000 A	26-06-2000
			BR	9915982 A	11-09-2001
			CN	1329674 T	02-01-2002
			WO	0034511 A2	15-06-2000
			EP	1137810 A2	04-10-2001
			JP	2002531141 T	24-09-2002
			US	2001034884 A1	25-10-2001
			ZA	200104490 A	04-06-2002
US 2002025508	A1	28-02-2002	DE	10000161 A1	19-07-2001
			WO	0149871 A2	12-07-2001
WO 0162897	A	30-08-2001	AU	3741201 A	03-09-2001
			WO	0162897 A1	30-08-2001
			EP	1257633 A1	20-11-2002
US 6333167	B1	25-12-2001	NONE		
WO 0206306	A	24-01-2002	AU	7795001 A	30-01-2002
			WO	0206306 A2	24-01-2002
			US	2003017991 A1	23-01-2003
WO 9813488	A	02-04-1998	DE	19641180 A1	26-03-1998
			AU	4775797 A	17-04-1998
			WO	9813488 A2	02-04-1998
			ZA	9708557 A	26-03-1998
DE 19920514	A	16-11-2000	DE	19920514 A1	16-11-2000
			WO	0068416 A2	16-11-2000
			EP	1177313 A2	06-02-2002
			JP	2002543797 T	24-12-2002